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Sex-specific effects of postnatal testosterone on lateralization in cichlid fish

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Lateralization is a fundamental principle in the organization of brain and behaviour in humans and nonhuman animals. To what extent lateralization is, in addition to genetic factors, under the influence of testosterone, which would also explain sex differences in laterality, is the topic of a long-standing debate. This debate is partly hampered by confusion between organizational and activating effects of testosterone. Here we focused on activating effects, less often studied than organizational effects. Studies on humans have shown ambiguous results and few experimental studies on animals have been conducted. We studied *Aequidens rivulatus*, a cichlid species in which lateralization of visually guided behaviour has been demonstrated and related to aggressiveness. After treatment with testosterone, lateralization was tested in a rotational preference task where fish had to face a predator. Testosterone induced significant lateralization at the population level. Testosterone-treated fish watched a predator preferentially with the right eye, which is in line with the literature on population-level lateralization of predator viewing in fish. It has been suggested that species differences in laterality of predator escape are related to sociality and we speculate that, within species, testosterone may affect lateralization in relation to a change in sociality. Among the fish of known sex, only males reacted to the treatment; a greater responsiveness of males may explain sex differences in lateralization found in many animal species. This may be caused by sex-specific sensitivity to androgens, perhaps in the habenular area of the brain.

Cerebral lateralization, the asymmetric distribution of functions over the two hemispheres, is a widespread phenomenon among vertebrates and results in lateralization of behaviour. Several studies have shown that males show a different lateralization pattern to females (humans: Hiscock et al. 1994; Wisniewski 1998; birds: Adret & Rogers 1989; fish: Brown et al. 2007; Reddon & Hurd 2008; reviewed in Pfannkuche et al. 2009) and some authors have suggested that this difference arises because of differences in exposure to testosterone (Wisniewski 1998), either prenatally or later in life, which has organizational (Schwarz & Rogers 1992; Rogers & Rajendra 1993; Sanders et al. 2002) or activating effects (Diamond 1991; Sanders et al. 2002) on lateralization.

Most attention so far has focused on the potential organizational nature of pre- and perinatal testosterone on lateralization (reviewed in Pfannkuche et al. 2009). However, activating effects may be equally relevant, for two reasons. First, some of the organizational effects of testosterone on brain lateralization may come about because of organizational effects on sex-specific production and sensitivity to androgens later in life. Second, organisms need to make constant adjustments to changes in their internal state and external environment, which may facilitate changes in lateralization during their lifetime. Since it has been suggested that lateralization at the population level is especially adapted for social behaviour (Vallortigara & Rogers 2005), and many aspects of social behaviour are under the influence of androgens, these hormones may be involved in inducing changes in lateralization during the animal's lifetime. Unfortunately, the activating effects of testosterone have been studied far less extensively than organizational effects and hypotheses about how testosterone affects lateralization in the adult brain are lacking.

It is well known that testosterone production is sensitive to diurnal rhythm (Plymate et al. 1989; Dabbs 1990), seasonal rhythm (Dabbs 1990; Meriggiola et al. 1996; Garde et al. 2000) and social cues (Oliveira 2004), which facilitates investigating the relationship between testosterone levels within their natural range and performance of lateralized functions, such as spatial skills. These studies suggest that testosterone suppresses spatial skills and therefore perhaps lateralization (Gouchie & Kimura 1991; Kimura & Toussaint 1991; Moffat & Hampson 1996). However, these studies are correlative and causal relationships are as yet unclear. Contrary to the correlative studies, experimental studies in humans, in which older males or female-to-male transsexuals received testosterone therapy, indicate that testosterone increases scores on spatial cognition (Janowsky et al. 1994; Slabbekoorn et al. 1999; van

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Goozen et al. 2002; Gooren & Giltay 2008; but see Sommer et al. 2008; reviewed in Cherrier 2009).

Spatial ability as such, however, is not a direct measure of lateralization. A few studies have analysed the relation between circulating levels of testosterone in human adults and handedness, a more direct measurement of lateralization. Results from these studies are, however, ambiguous too: Tan (1991) found higher levels of circulating testosterone in left-handers, ambidexters and right-handers with familial sinistrality than in right-handers, whereas Gadea (2003) and Moffat & Hampson (1996) found lower levels in left-handers. Other studies found no relation between testosterone and handedness (Moffat & Hampson 2000; Beaton et al. 2010). All of these studies assumed that individual differences in testosterone levels in adulthood reflect organizational effects of the hormone early in life. However, it is equally likely that these studies actually tested activating effects of testosterone.

Clearly, results of studies on the activating effects of androgens on human lateralization are ambiguous and call for experiments to be conducted on a random, nonpathological sample, representative of the population, using a direct measure of lateralization. Obviously, such experiments are more attainable in nonhuman animals than in humans, but are rare. Rogers et al. (1985) found that chickens, *Gallus gallus domesticus*, treated with testosterone early in life showed increased levels of attack when using their left eye but not when using their right eye, but whether this was an organizational or activating effect remains unclear. Del Rio-Portilla et al. (1997) found that in rats, *Rattus norvegicus*, gonadectomized after puberty, gonadal steroids were necessary to maintain sex differences in asymmetry shown in an EEG and a correlational study in primates showed that testosterone levels during adolescence were related to hand preference (Westergaard et al. 2000).

The evidence that testosterone has activating effects on lateralization is supported by indirect evidence from a recent study on cichlid fish. Adult fish showed a sex difference in lateralization, which correlated with their level of aggressiveness (Reddon & Hurd 2008). As aggressiveness and testosterone levels are correlated in fish (Munro & Pitcher 1985; Higby et al. 1991) the results suggest that testosterone activates sex differences in lateralization.

We experimentally tested the effect of testosterone on lateralization in male and female cichlid fish, expecting to find a similar interaction effect between sex and hormone treatment as Reddon & Hurd (2008) found between sex and aggressiveness. Fish have laterally placed eyes with small overlapping visual fields, which facilitates behavioural testing, easily revealing the lateralization pattern of the individual fish. In fish, predator inspection and predator viewing are lateralized (Bisazza & Vallortigara 1997; Bisazza et al. 1999; Brown et al. 2004): the fish preferentially inspect and view the predator using the right eye. Hence, we used predator viewing to investigate the effects of postnatal testosterone on the strength and direction of lateralization.

METHODS

Subjects and Housing

Aequidens rivulatus (a schooling, sexually dimorphic, substrate-breeding cichlid from Ecuador and Peru; Stawikowski & Werner 1998) were bred at our laboratory. These fish, like most cichlids, show elaborate display behaviour during agonistic and sexual interactions and are the subjects of a long-term study of behavioural lateralization in our department. Fish were reared in large stock aquaria (100 × 50 cm and 50 cm high), of which the back and sides were covered with black plastic, and fed dry fish flakes daily (TetraMin Tropical Fish Flakes).

Astronotus ocellatus, a South American predatory fish, were obtained from a local pet store. They were held in pairs in 200-litre tanks and were kept in the laboratory after the study. They were fed dry fish flakes daily and additionally received frozen shrimp weekly.

Fish were held in recirculating water filtration systems at 24 ± 2 °C and on a 12:12 h light:dark cycle.

Hormone Treatment

We used fish at 6 months old, the age at which they are about to become sexually mature. We used this age for two reasons. First, this is long after the period of sexual differentiation in cichlid fish (Pandolfi et al. 2006), confirmed in this species in our laboratory (A. F. H. Ros & T. G. G. Groothuis, unpublished data), so that exposure to testosterone was unlikely to have organizational effects. Second, the fish were not yet sexually active and did not show the nuptial sexually dimorphic coloration. Hence, endogenous testosterone levels in males were not yet elevated, enabling us to use control fish without the need for castration, which may influence the physiology of the animal dramatically.

At the start of the experiment, 120 fish (standard length: mean + SD = 26.79 + 3.49 mm) were distributed equally over the 12 tanks (35 × 24 cm and 24 cm high), half of which contained methyl-testosterone (Sigma), 0.5 mg dissolved in 0.0125 ml ethanol per litre water (a concentration shown to induce nuptial coloration and adult-like aggressive and sexual behaviour in *A. rivulatus*: Groothuis 1993), and half of which contained the solvent only, 0.0125 ml ethanol per litre water. Twice a week, we removed most of the water, leaving only a small layer, and replaced it with clean water, followed by renewal of the hormone treatment. Treatment lasted until the behavioural test started. For logistical reasons, only a limited number of fish could be tested each day, resulting in a variable treatment period of 8–12 weeks. This period of exposure to treatment was chosen based on a previous study (A. F. H. Ros & T. G. G. Groothuis, unpublished data) showing that juvenile fish show adult coloration and adult-like agonistic and courtship behaviour after this period of testosterone treatment and that the effects on both coloration and behaviour disappear after cessation of treatment.

Lateralization Test

We used a rotational preference test to investigate the lateralization of the fish. The apparatus consisted of a circular tank (diameter 30 cm) with a Plexiglas cylinder (diameter 12.5 cm) containing a predator (*A. ocellatus*) in the middle. To avoid possible lateralized interactions between the predator and the subject fish, the predator was restrained from moving by means of two Plexiglas plates. The tank was filled with 15 cm of water and was lit from above with a 60 W light bulb in the middle. Two sets of apparatus were used and placed in a completely darkened room. Before testing, the subject fish was placed in a small opaque tube, which was positioned anterior to the predator, in the darkened apparatus. After a 2 min acclimatization period, the light was switched on, the small opaque tube was gently removed from the tank and behaviour was videorecorded for 15 min. Four adult *A. ocellatus* (standard length: mean + SD = 9.88 + 0.47 cm) were used as predator stimuli. The recordings were analysed using the software program The Observer (Noldus Information Technology, Wageningen, The Netherlands). Time spent swimming in a clockwise or anticlockwise position was computed. We excluded from analysis the time fish spent turning to change their viewing position or trying to escape from the apparatus (facing the outer wall). Following the literature, we calculated a laterality index using the formula (time

anticlockwise − time clockwise)/(time anticlockwise + time clockwise) × 100. Positive values thus indicate more left-eye usage, whereas negative values indicate more right-eye usage when observing a predator. To investigate the strength of lateralization, independently of the direction, the absolute values of the laterality index were also computed.

Sex Determination

After euthanasia (see Ethical Note), the fish were weighed (to the nearest 0.1 g), measured (standard length to the nearest 1 mm) and decapitated. Fish were immediately frozen at −80 °C. At the time of histology the tail and epaxial musculature were removed and transverse serial sections were cut at 7–10 μm, stained with haematoxylin-eosin Y and examined under a light microscope. We scored the presence or absence of ovarian or testicular tissue. Hermaphrodite fish were not found.

Statistics

The laterality index and the log-transformed absolute laterality index were normally distributed and the variances of the dependent variables were not significantly different across groups. The laterality index and absolute laterality index were analysed using hierarchical linear models in the software program MLwiN 2.02 (J. Rasbash, C. Charlton, W. Browne, M. Healy, & B. Cameron, http://www.cmm.bristol.ac.uk/MLwiN/) to accommodate the hierarchical structure of our data set. We created two levels, tank being the highest and fish identity the lowest level. We fitted the models with treatment, sex and their interaction effect as categorical predictive factors. The models were estimated using restricted iterative generalized least squares (RIGLS). We tested the significance of the fixed factors using the Wald statistic, which follows a chi-square distribution.

To test whether groups showed a population bias of lateralization, we conducted one-sample t tests using the software package SPSS 16.0 (SPSS Inc., Chicago, IL, U.S.A.). In addition, to investigate whether differences in duration of exposure to testosterone affected the laterality index, a linear regression analysis was performed using SPSS 16.0 (http://www.gnu.org/software/pspp/). All tests were two tailed and significance levels were set to $\alpha < 0.05$.

Ethical Note

The testosterone or control treatment to which the experimental fish were exposed did not have any notable effects on their welfare. No adverse effects of testosterone were seen on survival or growth. Fish were killed by an overdose of MS-222 (1 g/litre) buffered with sodium bicarbonate. Opercular movement ceased within 1 min and fish were left in the solution additionally for at least 10 min. We used MS-222 in more than five times the anaesthetic solution (150 mg/litre for induction in cichlids, Neiffer & Stamper 2009) as recommended by the American Veterinary Medical Association (2007).

The predators, being hand tame and very calm in our tanks, were restrained during the behavioural trials by means of two Plexiglas plates to prevent any lateralized interactions between the predator and the subject fish. These plates were loosely placed in such a manner that the fish could still move but could not turn. The predators showed tonic immobility when first placed in this position, but after approximately 2 min showed a normal posture and no changes to a darker colour (a sign of stress in many cichlid fish) were noted. Individual *A. ocellatus* were used for a maximum time of 1 h and reused after a 47 h period of recovery in its home tank. All experimental protocols were approved by the ethical committee for animal research of the University of Groningen.

RESULTS

Consistent with our expectation, fish treated with testosterone showed a population bias to look at a predator with the right eye, thus using the left hemisphere (one-sample t test: $t_{19} = -2.268$, $P = 0.035$), whereas in the control treatment no such bias was found (one-sample t test: $t_{18} = 0.181$, $P = 0.858$; Fig. 1a). Testosterone treatment did not affect the absolute value of the laterality index (Wald $\chi^2_1 = 0.071$, $P = 0.790$; Fig. 1b).

Histological examination revealed 19 females (nine in control group, 10 in testosterone group), 10 males (five in each group) and 10 fish of which the sex could not be determined (five in each group) as no gonadal tissue was yet apparent. Testosterone affected the laterality index of males differently to that of females and this interaction effect between treatment and sex was significant (Wald $\chi^2_3 = 9.234$, $P = 0.026$; Fig. 2a). Post hoc tests showed that testosterone-treated males differed significantly from control males (Wald $\chi^2_1 = 5.427$, $P = 0.020$). No other post hoc tests were significant. Testosterone treatment did not affect the absolute value of the laterality index of sex-determined fish (interaction treatment*sex: Wald $\chi^2_3 = 2.180$, $P = 0.536$; treatment: Wald $\chi^2_1 = 0.066$, $P = 0.797$; sex: Wald $\chi^2_1 = 2.154$, $P = 0.142$; Fig. 2b).

Within the testosterone-treated group, there was no effect of the variation in duration of testosterone treatment on the laterality index ($\beta = -0.19$, $t_{17} = -0.836$, $P = 0.414$, $R^2 = 0.037$).

DISCUSSION

We tested the effect of testosterone on visually guided behavioural lateralization in fish just before sexual maturity and found a clear effect in males, but not in females. In addition, testosterone induced lateralization at the level of the population. These fish preferentially viewed the predator with the right eye, thus using the left hemisphere. These findings are in line with the literature and our expectations.

The direction of lateralization we found in fish treated with testosterone is in accordance with results obtained from similar tests of several fish species (Bisazza & Vallortigara 1997; Bisazza et al. 1999). Studies have shown that a bias to turn left, thus fixating on the predator with the right eye, occurs when fish are escaping from a predator (Cantalupo et al. 1995; Lippolis et al. 2009). A bias in the direction of the escape response within fish shoals is thought to be adaptive to a certain degree as it hampers the predator's attempts to catch its prey owing to a dilution effect in shoaling animals (Vallortigara & Rogers 2005).

This intraspecific variation in eye use resulting from the hormone treatment may be related to the explanation for interspecific variation in eye use that is based on interspecific variation in the tendency to shoal (Bisazza et al. 2000), which may also vary with predation pressure and other environmental variables (Brown & Warburton 1997). In shoaling species, individuals must be able to monitor both predators and shoalmates simultaneously. Selection for such lateralization might be lacking in nonshoaling fish, but present in shoaling fish. It is intriguing that lateralization at the level of the population is seen as an adaptation for social behaviour (Vallortigara & Rogers 2005), that many social behaviours are under the influence of testosterone (Nelson 2005), and that we found an effect of testosterone on lateralization. Perhaps within species, testosterone induces a change in life history stage from being more solitary to becoming more social, inducing fish to come together on the breeding grounds, making synchronous escape movements away from predators beneficial.

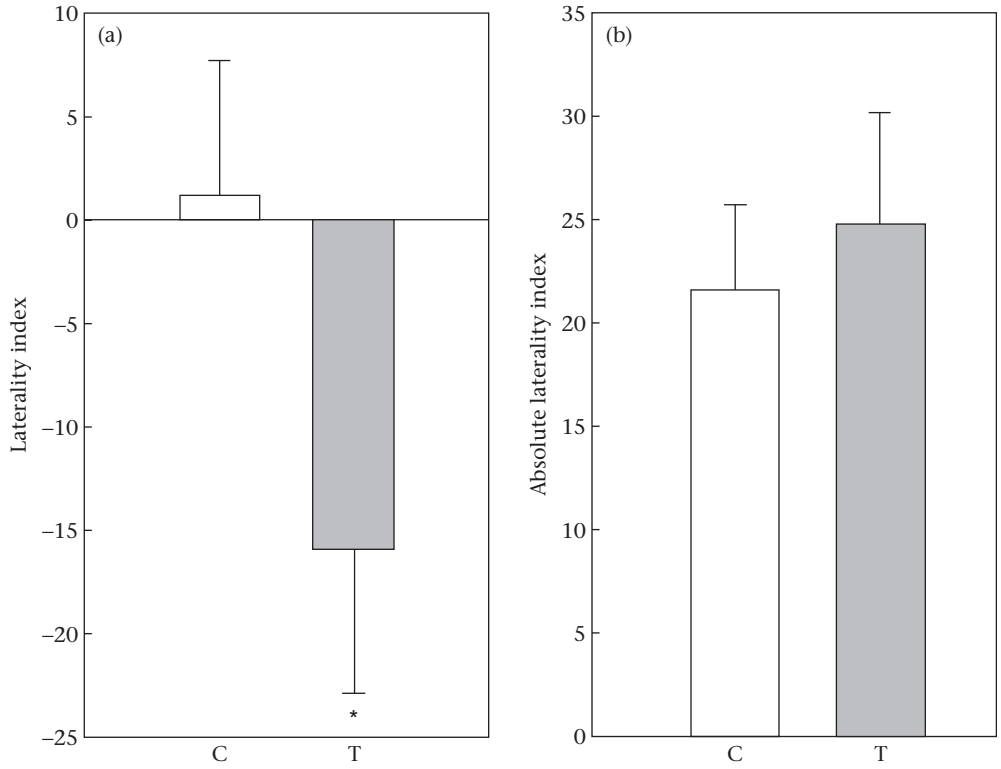

Figure 1. (a) Laterality index of the rotational test in the control (C; white bars, $N = 19$) and testosterone treatment (T; grey bars, $N = 20$) groups. Positive values indicate more left-eye usage, negative values indicate more right-eye usage. (b) Absolute laterality index of the rotational test in the control and testosterone treatment groups. Means are shown + SEs. $^*P < 0.05$.

Testosterone had different effects on males and females. This sex effect highlights the importance of studying lateralization with respect to the sex of the animal. Reddon & Hurd (2008) found an interaction effect between aggressiveness and sex on lateralization similar to our findings. Testosterone may be the underlying mechanism causing this result, as testosterone levels and aggressiveness are positively related in fish (Munro & Pitcher 1985; Higby et al. 1991).

The finding that males were more sensitive to testosterone than females is consistent with other studies in many vertebrates and probably caused by the early organizational effects of steroids on sexual differentiation, for example by its effect on the synthesis of

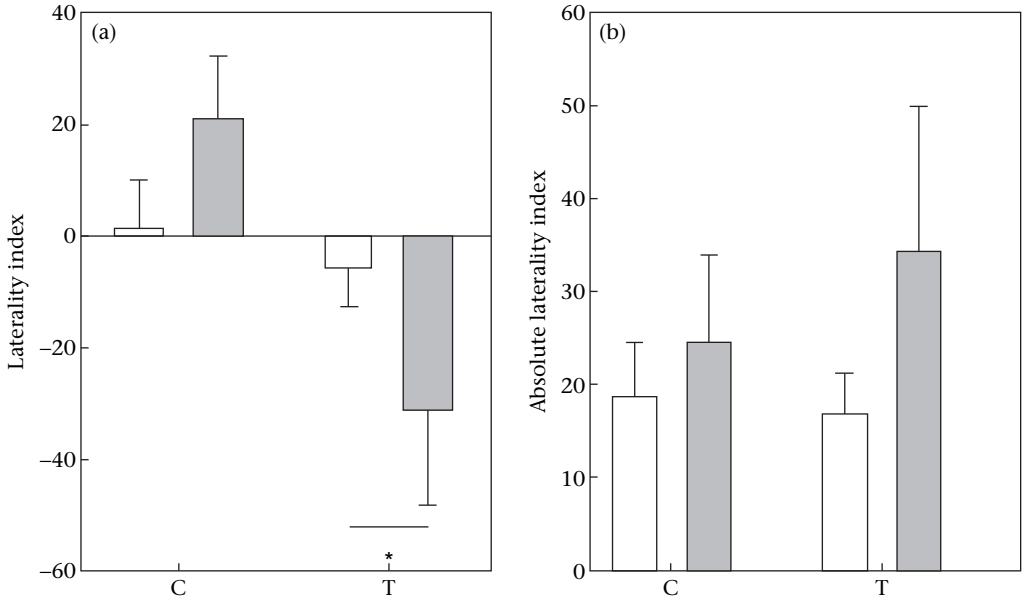

Figure 2. (a) Laterality index of the rotational test for females (white bars, $N_{control} = 9$, $N_{testosterone} = 10$) and males (grey bars, $N_{control} = 5$, $N_{testosterone} = 5$) in the control (C) and testosterone treatment (T) groups. (b) Absolute laterality index of the rotational test for females and males in the control and testosterone treatment groups. Means are shown + SEs. $^*P < 0.05$.

androgen receptors (Nelson 2005). Our results open the possibility that behavioural lateralization is partly induced by sex-specific lateralization of androgen receptors but as yet this has received no attention in the literature.

Furthermore, testosterone is known to have sex-specific effects on the structural asymmetry of the habenular nucleus in the vertebrate brain: testosterone treatment changed the asymmetry of female chickens to that of males when applied soon after hatching (Gurusinghe et al. 1986). This nucleus is the best-known example of brain asymmetry (Concha & Wilson 2001). In frogs, both males and females show asymmetries of the dorsal habenula, and these asymmetries are more pronounced in spring, the mating season, than in winter, indicating a modulating role for activating effects of testosterone on the habenula (Kemali et al. 1990).

All the effects of testosterone that we found concerned the direction and not the strength of lateralization. Assuming that testosterone regulates aggression, our results are therefore inconsistent with the finding that sex and aggressiveness interact in affecting the strength of lateralization in another cichlid species (Reddon & Hurd 2008). However, the translation of their results to ours may be too simplistic, as aggression is not only a reflection of levels of testosterone and testosterone does not only regulate aggression.

In conclusion, the predominant genetic models concerning lateralization are limited in their explanatory power and leave scope for environmental factors such as gonadal steroids to affect lateralization patterns (Schaafsma et al. 2009). However, this potential hormonal effect is still heavily disputed. Although we cannot entirely rule out the possibility that the effect of testosterone treatment that we found might have been both activating and organizational, our study showed that lateralization is a plastic trait and that testosterone affects lateralization well after the pre- and perinatal period. Males were more responsive to exogenous testosterone than females in the effect on lateralization, and this difference in responsiveness may explain sex differences in lateralization that have been demonstrated in several species. Our results therefore provide a model to analyse the activating effect of testosterone in neurobiology, an enterprise so far receiving much more attention for the study of organizational effects of the hormone on lateralization.

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